JIPB Journal of Integrative Plant Biology

Mitochondrial GPX1 silencing triggers differential photosynthesis impairment in response to salinity in rice plants

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Research Article

Abstract The physiological role of plant mitochondrial glutathione peroxidases is scarcely known. This study attempted to elucidate the role of a rice mitochondrial isoform (GPX1) in photosynthesis under normal growth and salinity conditions. GPX1 knockdown rice lines (GPX1s) were tested in absence and presence of 100 mM NaCl for 6 d. Growth reduction of GPX1s line under non-stressful conditions, compared with non-transformed (NT) plants occurred in parallel to increased H_2O_2 and decreased GSH contents. These changes occurred concurrently with photosynthesis impairment, particularly in Calvin cycle's reactions, since photochemical efficiency did not change. Thus, *GPX1* silencing and downstream molecular/metabolic changes modulated photosynthesis differentially. In contrast, salinity induced reduction in both phases of photosynthesis, which were more impaired in silenced plants. These changes were associated with root morphology alterations but not shoot growth. Both studied lines displayed increased GPX activity but H_2O_2 content did not change in response to salinity. Transformed plants exhibited lower photorespiration, water use efficiency and root growth, indicating that GPX1 could be important to salt tolerance. Growth reduction of GPX1s line might be related to photosynthesis impairment, which in turn could have involved a cross talk mechanism between mitochondria and chloroplast originated from redox changes due to GPX1 deficiency.

Keywords: CO_2 assimilation; glutathione peroxidase; *Oryza sativa*; photochemistry; redox homeostasis
Citation: Lima-Melo Y, Carvalho FEL, Martins MO, Passaia G, Sousa RHV, Neto MCL, Margis-Pinheiro M, Silveira JAG (2016) Mitochondrial GPX1 silencing triggers differential photosynthesis impairment in response to salinity in rice plants. **J Integr Plant Biol** XX:XX–XX doi: 10.1111/jipb.12464
Edited by: Miguel A. Piñeros, United States Department of Agriculture-Agricultural Research Service, Cornell University, USA
Received Dec. 22, 2015; **Accepted** Jan. 19, 2016
Available online on Jan. 22, 2016 at www.wileyonlinelibrary.com/journal/jipb

INTRODUCTION

Glutathione peroxidases (GPX, EC 1.11.1.9) are largely distributed in animal cells, where they play several important physiological roles such as H_2O_2 scavenging, maintaining of cellular redox homeostasis and membrane protection against peroxidation (Herbette et al. 2007). Plants also have a large family of GPX isoforms distributed in virtually all cellular compartments (Margis et al. 2008; Passaia and Margis-Pinheiro 2015). Differently from animal GPX, the physiological role of these peroxidases in plants is less known (Faltin et al. 2010; Gaber et al. 2012; Zhai et al. 2013). Despite the several experimental evidences that have suggested that plant GPX family displays some similar roles of those played in animals, the physiological functions of specific isoforms are still unknown (Passaia et al. 2014a; Passaia and Margis-Pinheiro 2015). These enzymes might act as antioxidants in the elimination of H_2O_2, organic hydroperoxides and/or lipid peroxides (Faltin et al.

Abbreviations

A	CO_2 assimilation rate
A_{max}	maximum photosynthetic rate
C_i	intercellular CO_2 partial pressure
E	leaf transpiration
ETR	actual electron transport rate from PSII
F_v/F_m	maximum quantum yield of PSII
GPX	glutathione peroxidase
g_s	stomatal conductance
GSH	reduced glutathione
J_{max}	maximum electron transport rate
L_s	stomatal limitation
L_m	metabolic limitation
NPQ	non-photochemical quenching
PPFD	photosynthetic photon flux density
P_r	photorespiration
PSII	photosystem II
qP	photochemical quenching
R_d	dark respiration
ROS	reactive oxygen species
TBARS	thiobarbituric acid reactive substances
Vc_{max}	maximum carboxylation rate of Rubisco
WUE	water use efficiency
α	quantum efficiency
$\Delta F/F_m'$	effective quantum yield of PSII

2010; Passaia et al. 2013). In addition, as GPX might use thioredoxins and GSH as reducing substrates (Chang et al. 2009), this action *per se* could contribute to change the redox homeostasis in plant cells especially under stress conditions.

The preferential utilization of oxidizing and reducing agents by this enzyme as substrates under *in vivo* conditions has been controversy. Some evidences have suggested that thioredoxins and hydroperoxides are favored in comparison to GSH and H_2O_2 (Herbette et al. 2002; Passaia and Margis-Pinheiro 2015) while other studies have demonstrated that GPX may use GSH and H_2O_2 as substrates (Chang et al. 2009; Foyer and Noctor 2011). The action of the GPX family in plants, like in animals, could be important to prevent or restrict H_2O_2 accumulation, protect membranes against lipid peroxidation and act in cellular signaling (Foyer and Noctor 2011). Several studies have demonstrated that GPX, especially chloroplast isoforms, are important in the protection against several abiotic and biotic stresses (Roxas et al. 2000; Yoshimura et al. 2004; Miao et al. 2006). Some studies have demonstrated that transgenic *Arabidopsis* plants over-expressing chloroplastic GPX are more resistant to abiotic and biotic stresses (Roxas et al. 2000; Yoshimura et al. 2004) and mutants carrying knockout constructions in these genes are more sensitive to some stresses (Chang et al. 2009; Gaber et al. 2012).

Miao et al. (2006) demonstrated that *Arabidopsis* GPX3 is involved in the stomata control and this mechanism is related with a balance between H_2O_2 and ABA levels. Moreover, *Arabidopsis* transformed plants lacking chloroplastic GPX exhibited severe morphological, anatomical and functional alterations in leaves (Chang et al. 2009), demonstrating the physiological importance of this protein. Recently, it has been shown that the GPX proteins are also important in the control of root architecture in *Arabidopsis* (Passaia et al. 2014b). Thus, there is a relative consensus on the physiological importance of chloroplast GPX. However, despite *Arabidopsis* plants enclose eight GPX genes (Margis et al. 2008), the reported studies have concentrated mainly on chloroplast isoforms (Yoshimura et al. 2004; Chang et al. 2009). Working with GPX isoforms of rice, our group has demonstrated that the mitochondrial isoforms, GPX1 and GPX3, are both important for growth, development and H_2O_2 homeostasis (Passaia et al. 2013; Passaia et al. 2014a). Recently, it has been proposed that plant GPX isoforms could act as redox sensor proteins in signal transduction in addition to their peroxidase activity (Passaia and Margis-Pinheiro 2015). Nevertheless, the physiological roles of plant mitochondrial GPX are still scarcely known (Jacoby et al. 2011).

Rice has five identified GPX isoforms: GPX1 and GPX3 (mitochondrial), GPX2 and GPX5 (cytosolic) and GPX4 (chloroplastic) (Margis et al. 2008), but both cytosolic isoforms were also found in chloroplasts (Passaia et al. 2013). Mitochondrial GPX1 is important for rice growth and spikelet formation whereas mitochondrial GPX3 modulates plant development and H_2O_2 homeostasis in roots (Passaia et al. 2013, 2014a). H_2O_2 is a central signaling molecule involved in several processes that connects metabolism between plant cell organelles (Sewelam et al. 2014). Despite several experimental studies having demonstrated that the photosynthesis and respiration are tightly interconnected (Sweetlove et al. 2006; Zhang et al. 2012), studies on the role of plant mitochondrial GPX and other antioxidant enzymes in a H_2O_2-GSH mediated cross talk involving these processes are lacking. In C3 plants like rice, mitochondria, chloroplasts and peroxisomes are closely associated in metabolic terms during photorespiration, especially under stress conditions (Munné-Bosch et al. 2013).

The literature concerning the physiological role of mitochondrial GPXs in plants is poorly known. GPX1 could contribute to redox homeostasis in mitochondria, acting as peroxidase and modulating the levels of H_2O_2, GSH and thioredoxins (Passaia and Margis-Pinheiro 2015). The effects of salinity on expression and activity of mitochondrial GPXs are less known. Salinity strongly reduces growth and alters redox homeostasis in mitochondria, changing H_2O_2 levels and inducing oxidative stress (Cavalcanti et al. 2007). Correspondingly, salt excess causes generalized effects in virtually all plant cells and organelles, but the knowledge of specific consequences on the redox metabolism in mitochondria is limited (Milla et al. 2003; Mittova et al. 2003; Cavalcanti et al. 2007). In photosynthesis, the effects of salinity are well known especially indirect consequences on the stomatal conductance, photochemical and Calvin cycle reactions (Munns and Tester 2008; Chaves et al. 2009). Some reports in *Arabidopsis* have evidenced that chloroplastic GPX isoforms are important to regulation of redox homeostasis and protection against oxidative stress generated by salinity (Milla et al. 2003). However, the role of mitochondria GPX on chloroplast redox metabolism is unknown. As salt stress simultaneously affects growth, antioxidant metabolism and photosynthesis in rice (Bonifacio et al. 2011), this model was chosen to evaluate the role of a mitochondrial GPX in photosynthesis efficiency, in two distinct circumstances: normal condition, where a lack of that enzyme affects rice growth, and salt stress, which induces strong reduction in photosynthesis and plant growth.

In this study, we tested the hypothesis that reduced growth in rice plants with decreased expression of the mitochondrial isoform GPX1 involves the impairment of photosynthesis. In addition, as salinity strongly decreases growth, photosynthesis and induces disturbances in the redox homeostasis, we investigated the possibility that the importance of GPX1 on plant growth could be intensified under this abiotic stress. The decreased growth and development in rice deficient in GPX1 under normal conditions was associated with impairment in photosynthesis caused by restriction in the Calvin cycle reactions but not in photosystem II activity. In contrast, salinity induced reduction in both phases of photosynthesis, and these decreases were more intense in silenced plants. The effects of GPX1 deficiency on photosynthesis involving a possible cross talk between mitochondria and chloroplasts are discussed.

RESULTS

Phenotype and physiological characterization of mitochondrial GPX1 silenced plants

In a previous study, the phenotyping of eight GPX1s lines was performed and demonstrated that GPX1 knockdown induced significant phenotypic alterations indicated by significant reduction in growth and seed yield (Passaia et al. 2013). The three selected lines used in this study have exhibited reductions in the transcript amounts by 71%, 73% and 61%

compared with non-transformed (NT) plants (Figure S1). These knockdowns were sufficient to trigger reductions in growth as indicated by decreased shoot fresh weight in all three lines compared with NT plants (Table S1). The reduced growth exhibited the same trend as CO_2 assimilation rate (*A*) and water use efficiency (WUE), which was calculated by the quotient between net CO_2 assimilation and net transpiration. The photosystem II (PSII) efficiency parameters, such as actual quantum yield ($\Delta F/F_m'$) and electron transport rate (ETR) in knockdown lines were similar to the values found in NT plants (Table S1).

Low GPX1s growth was related to increased H_2O_2, decreased GSH and unchanged levels of lipid peroxidation

As the three selected GPX1s lines exhibited similar physiological characteristics (photosynthesis and growth), the L29 line was selected as a representative GPX1 knockdown plant. Initially, we performed a growth analysis from the 15th day after germination (DAG) to the 48th DAG (during the vegetative phase) to verify any phenotypic alteration in silenced line. In this time interval, GPX1s showed slower relative growth, which was indicated by a lower slope of the shoot length rate curve (Figure 1A). Interestingly, the relative leaf emission rate (leaf number per plant) did not change, indicating no alteration in the development (Figure 1B). These changes were associated with the smaller leaf area exhibited by the GPX1 (data not shown). To determine if the GPX1 silencing could have triggered changes in some oxidant/antioxidant components, the contents of H_2O_2, GSH and TBARS (lipid peroxidation) were performed in leaves. The contents of H_2O_2 increased, GSH decreased and TBARS remained unchanged (Figure 2).

GPX1 was important for root development but not for membrane damage whereas GPX activity increased in both NT and GPX1s plants in response to salinity

48-d-old GPX1s and NT plants were exposed to 100 mM NaCl for 6 d to investigate whether the reduced growth of GPX1s

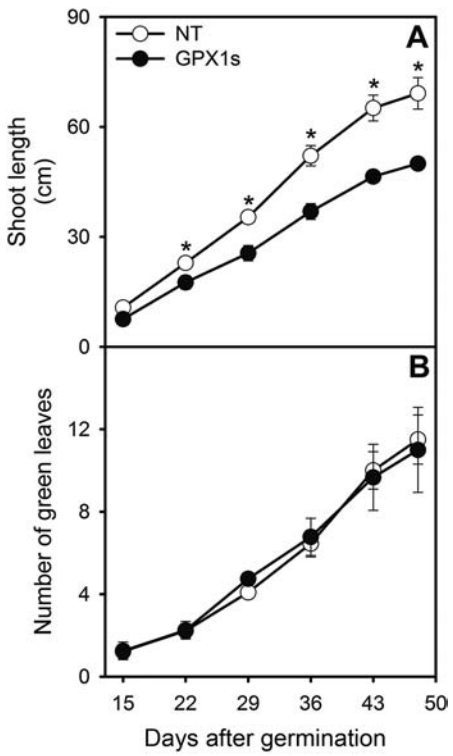

Figure 1. Phenotypic characterization of NT and *GPX1* silenced rice plants

Changes in shoot length (**A**) and number of leaves (**B**) of non-transformed (NT) and *GPX1* silenced rice plants during the vegetative growth stage. Each point represents the mean of ten replicates ± *SD*. Asterisks represent significant differences between the genotypes calculated by Tukey's test ($P < 0.05$).

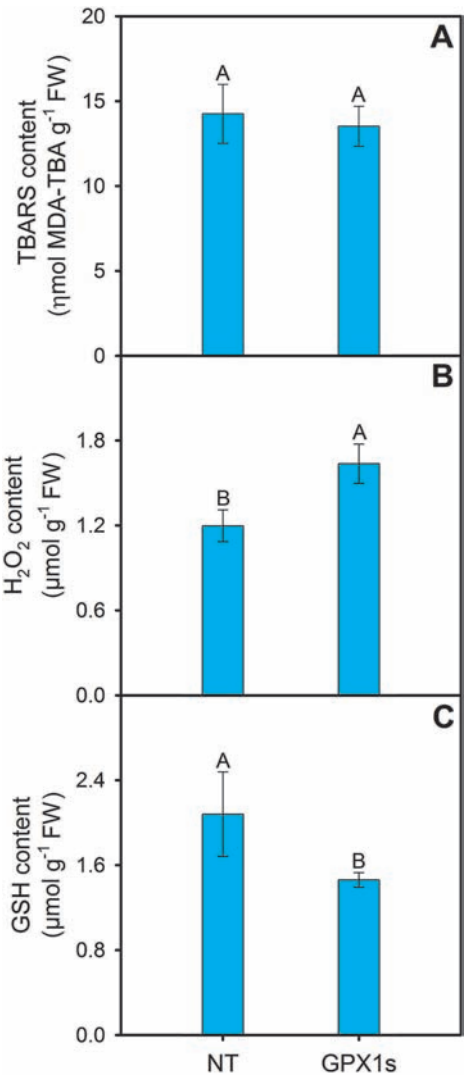

Figure 2. Oxidative stress indicators in NT and *GPX1* silenced rice leaves

Lipid peroxidation (TBARS) level (**A**), hydrogen peroxide content (**B**) and reduced glutathione content (**C**) in leaves of non-transformed (NT) and *GPX1* silenced rice exposed to growth conditions. The means are representative of three replicates ±*SD*. Capital letters represent significant differences between the genotypes calculated by Tukey's test ($P < 0.05$).

could be aggravated or attenuated under salt stress, since we found that this protein is important for growth modulation in non-stressful conditions. GPX1s exhibited lower shoot- and similar root-mass compared with NT in the absence of NaCl. In opposition, in the presence of salinity, GPX1s showed similar shoot- and lower root-mass, indicating that under these conditions GPX1 was important for root development but not for shoot growth (Figures 3, S2). In control conditions, both NT and silenced plants exhibited similar Na^+ content and membrane damage (indicated by electrolyte leakage) in leaves (Figure 4). Under salinity, both NT and GPX1s plants showed an increase in Na^+ content and electrolyte leakage, which were lower in GPX1s in comparison to NT (Figure 4). The leaf K^+/Na^+ ratios in both salt-stressed GPX1s and NT plants were approximately 1.80 (data not shown), indicating that both genotypes were exposed to a moderate salt stress. These physiological changes were related to increase in GPX activity in both genotypes whereas the leaf H_2O_2 content did not change by salt effect but remained higher in the GPX1s plants (Figure 5).

Mitochondrial isoform GPX1 differentially modulated the phases of photosynthesis in non-stressful and salinity conditions

Several photosynthetic parameters were calculated from the *A*-C_i and *A*-PPFD curves (Table 1; Figures 6, S3). The major photosynthetic efficiency indicators are shown in Table 1. The maximum carboxylation rate of Rubisco (Vc_{max}), an indicator of *in vivo* Rubisco activity, was lower in the transformed plants compared with NT in control conditions and it decreased in both studied plants, reaching similar values under salinity. The maximum electron transport rate (J_{max}) for RuBP regeneration rate, exhibited a similar trend to Vc_{max}. GPX1s presented lower stomatal limitation (L_s) and higher metabolic limitation (L_m) compared with NT under control conditions. The maximum photosynthesis (A_{max}), calculated from the *A*-PPFD curves, was lower in the silenced plants in both experimental conditions. The potential yield of photosystem II (F_v/F_m) was similar in both plant lines. The CO_2 quantum efficiency (α), which indicates the amount of assimilated CO_2 per incident photon, was higher in the NT plants compared with the silenced line in control conditions but not under salt stress. In addition, silenced plants exhibited lower day respiration (R_d) and photorespiration (P_r) in control conditions. Under saline conditions, the plant lines exhibited similar R_d values. The P_r values under salinity increased in both plant lines but remained lower in the GPX1 knockdown plants.

The CO_2 assimilation rates increased as the light intensity (PPFD) was increased in both treatments but NT displayed higher photosynthesis than GPX1s from 200 $\mu mol\, m^{-2}\, s^{-1}$ to 2,000 $\mu mol\, m^{-2} \cdot s^{-1}$. NT plants displayed higher photosynthesis compared with GPX1s in the two experimental conditions, practically in all PPFD values (Figure 6A). The PSII efficiency parameters ($\Delta F/F_m'$ and ETR) were similar in NT and GPX1s in all studied light intensities under non-stressful conditions (Figure 6B, C). Inversely, when these two lines were exposed to salinity, NT plants displayed higher values of $\Delta F/F_m'$ and ETR in all light intensities compared with GPX1s (Figure 6B, C). Non-transformed plants exhibited higher NPQ formation at light intensities higher than 100 $\mu mol\, m^{-2} \cdot s^{-1}$, under control conditions and compared with GPX1s (Figure 6D). In contrast, salt-treated plants of both lines presented significant increase and similar NPQ formation as the light intensity was increased, being similar in both genotypes. On the other hand, the gas exchange parameters revealed that GPX1s and NT plants presented similar values of stomatal conductance (g_s) and transpiration (*E*) in non-stressful conditions and salinity caused a strong decrease in these parameters in both plant lines (Figure 7A, B). GPX1s showed higher intercellular CO_2 partial pressure (C_i) values compared with NT plants in both control and salt treatment

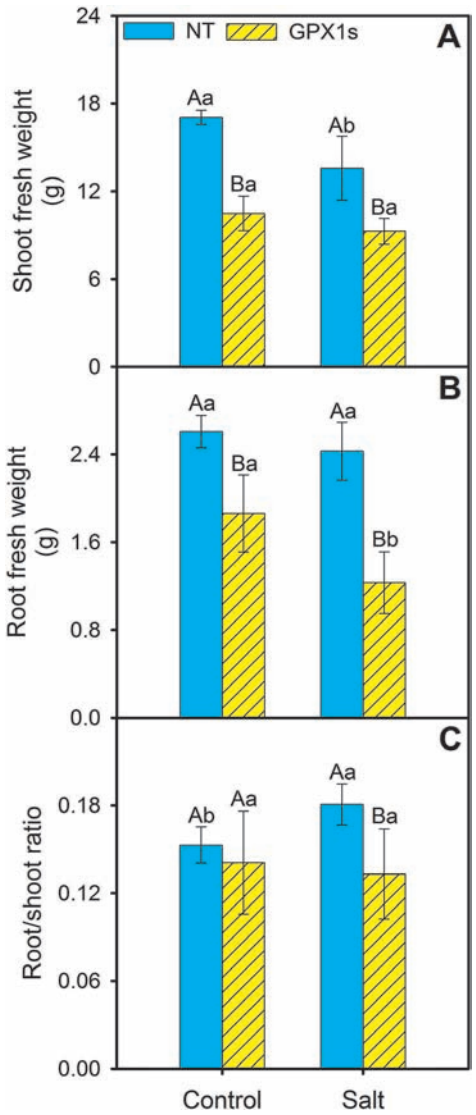

Figure 3. Effects of salt stress on fresh mass accumulation in NT and *GPX1* silenced rice plants

Shoot fresh weight (**A**), root fresh weight (**B**) and root/shoot fresh weight ratio (**C**) of non-transformed (NT) and *GPX1* silenced rice exposed to control conditions and 100 mM NaCl for 6 d. The means are representative of four replicates ± *SD*. Capital letters represent significant differences between genotypes within treatments, and lower case letters represent significant differences between treatments within genotypes, calculated by Tukey's test ($P < 0.05$).

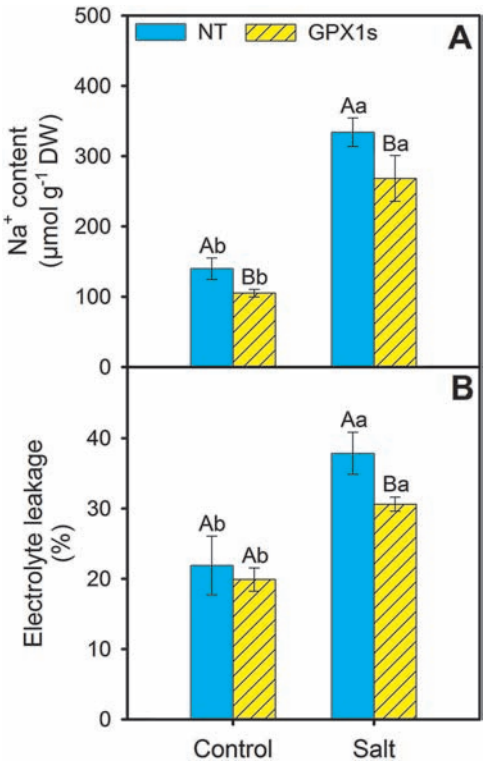

Figure 4. Salt stress and cellular indicators in NT and *GPX1* silenced rice leaves
Na^+ content (**A**) and electrolyte leakage (**B**) in leaves of non-transformed (NT) and *GPX1* silenced rice exposed to control conditions and 100 mM NaCl for 6 d. The means are representative of four replicates ± *SD*. Capital letters represent significant differences between genotypes within treatments, and lower case letters represent significant differences between treatments within genotypes, calculated by Tukey's test ($P < 0.05$).

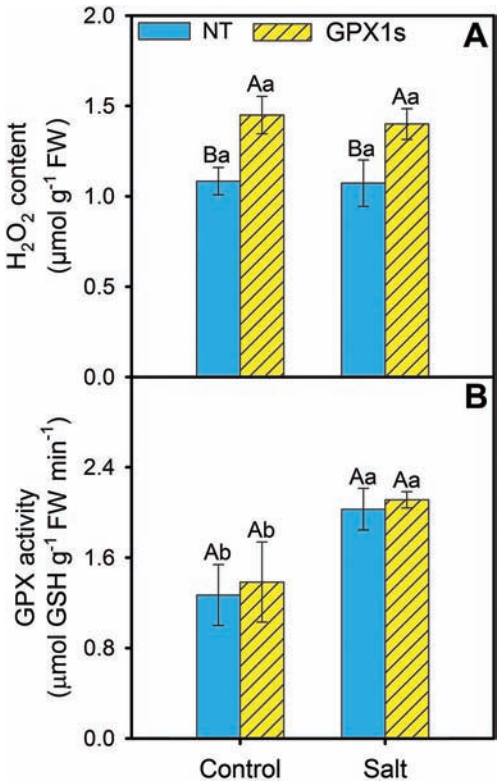

Figure 5. H_2O_2 content and total GPX activity in NT and *GPX1* silenced rice leaves
Hydrogen peroxide content (**A**) and GPX activity (**B**) in leaves of non-transformed (NT) and *GPX1* silenced rice exposed to control conditions and 100 mM NaCl for 6 d. The means are representative of four replicates ±*SD*. Capital letters represent significant differences between genotypes within treatments, and lower case letters represent significant differences between treatments within genotypes, calculated by Tukey's test ($P < 0.05$).

and salinity induced decrease in this parameter in the two plant lines (Figure 7C). As a consequence of decreased photosynthesis, GPX1s plants showed lower water use efficiency, in both non-stressing and salt stress conditions (Figure 7D).

DISCUSSION

The data demonstrate clearly that rice plants silenced in *GPX1* grown in normal conditions display a strong decrease in plant growth in parallel to a prominent impairment in photosynthesis. Interestingly, this photosynthetic restriction occurred only at level of Calvin cycle and related parameters, whereas PSII activity did not change. These plants displayed increased H_2O_2 and decreased GSH contents, two important redox components involved in several important physiological processes as signaling and metabolism regulation (Dietz 2014). Possibly these redox changes were direct or indirectly triggered by GPX1 deficiency in mitochondria and such alterations could have changed the metabolism in different levels and in distinct cellular compartments. Previously, we have demonstrated that in rice silenced in other mitochondrial GPX isoform (GPX3), the restriction in root growth was associated with H_2O_2 modulation (Passaia et al. 2014a). In addition, previously we also have demonstrated that the knockdown of *GPX1* does not substantially affect the expression of other GPX genes since the *GPX1*-silenced transgenic plants presented a reduction in the *GPX5* gene expression (cytosolic) of about 30% in comparison to NT plants, whereas the expression of the other *GPX* genes did not change (Passaia et al. 2014a). Therefore, we can discard that the results observed for the silencing of *GPX1* could be produced by a potential functional redundancy of the GPX family or, alternatively, by the silencing of other member of the GPX family by off-target silencing.

Despite the physiological role of mitochondrial isoforms of GPX in plants is practically unknown, GPX proteins might display important cellular roles such as H_2O_2 scavenging, membrane protection and as a redox modulator protein, as recently proposed (Passaia and Margis-Pinheiro 2015). Under *in vivo* conditions it has been proposed that GPX uses preferentially thioredoxins and organic hydroperoxide as substrates (Passaia et al. 2014a). However, some works have

Table 1. Photosynthetic parameters of non-transformed (NT) and *GPX1* silenced rice plants exposed to control and salinity conditions

	Control		Salt	
Measurements	NT	GPX1s	NT	GPX1s
Vc_{max}	116.1 Aa	102.2 Ba	60.2 Ab	55.8 Ab
J_{max}	146.7 Aa	119.1 Ba	67.3 Ab	62.7 Ab
L_s (%)	22.68 Ab	17.66 Bb	33.51 Aa	28.64 Aa
L_m (%)	0	14.34 B	46.53 Aa	53.52 Aa
A_{max}	19.6 Aa	13.7 Ba	10.2 Ab	7.9 Bb
F_v/F_m	0.755 Aa	0.766 Aa	0.806 Aa	0.798 Aa
α	0.042 Aa	0.027 Ba	0.033 Ab	0.025 Aa
R_d	1.080 Aa	0.324 Bb	1.346 Aa	1.325 Aa
P_r	2.56 Ab	2.05 Bb	4.12 Aa	2.91 Ba

Maximum carboxylation rate of Rubisco (Vc_{max}), maximum electron transport rate (J_{max}), stomatal limitation (L_s) and metabolic limitation compared to NT plants under the control condition (L_m), maximum photosynthetic rate (A_{max}), potential quantum efficiency (F_v/F_m), CO_2 quantum efficiency (α), dark respiration (R_d) and photorespiration (P_r) calculated from *A*-Ci and *A*-PPFD curves of non-transformed (NT) and *GPX1* silenced (GPX1s) rice exposed to control conditions and 100 mM NaCl for 6 d. The means are representative of four replicates $\pm$ *SD*. Capital letters represent significant differences between genotypes within treatments, and lower case letters represent significant differences between treatments within genotypes, calculated by Tukey's test ($P < 0.05$).

evidenced that GPX might also use, in a minor extent, GSH and H_2O_2 (Chang et al. 2009). In this study GPX activity increased significantly in both plant lines in response to salinity but the H_2O_2 content remained unchanged, suggesting that *in vivo* this peroxide was not used preferentially by GPX. Beside its peroxidase activity, GPX could interact with thiol proteins and H_2O_2, resulting interactions that would change the thiol-redox state, allowing cell signaling (Passaia and Margis-Pinheiro 2015). Frequently, other thiol-enzymes such as glutathione-S-transferase (GST), monodehydroascorbate reductase, peroxidase-type GST, GSH-dependent thioredoxin reductase and other reactions between GSH and thiol-proteins are direct or indirectly related to GPX, thioredoxins, GSH and H_2O_2 metabolism, with consequences in the modulation of complex processes (Dietz 2014).

The metabolic/molecular alterations triggered by GPX1 deficiency could have induced modifications in redox state of different cell compartments (Dietz 2014), since the levels of H_2O_2 and GSH were changed. Some works have evidenced that mitochondrial malate desidrogenase could act linking the energy exchange between mitochondria-cytosol-chloroplast (Munné-Bosch et al. 2013). The energy balance into mitochondria involves redox homeostasis (Hebelstrup and Møller 2015) but the role of mitochondrial GPX in this process is unknown. Some authors have postulated that H_2O_2, GSH and probably other signaling could act mediating this cross talking between organelles (Munné-Bosch et al. 2013). In this manner, mitochondrial GPX activity could participate in these mechanisms inducing changes in redox homeostasis and alterations on H_2O_2, GSH and thioredoxins levels or even acting as modulator protein as recently suggested (Passaia and Margis-Pinheiro 2015). Thus, the great difficulty to establish the possible role of mitochondrial GPX in photosynthesis metabolism is elucidating the genes and metabolic networks involved with these complex mechanisms. These problems are difficult even under normal growth conditions and, obviously, more complex in response to abiotic stress like salinity.

Rice plants deficient in mitochondrial GPX have exhibited low growth and triggered changes in H_2O_2 homeostasis in normal and under some abiotic stress conditions (Passaia et al. 2013). It is plausible to argue that this ROS could be involved in the growth regulation (Pnueli et al. 2003) and perhaps mediating cross talking between organelles and other cellular compartments (Sewelam et al. 2014). H_2O_2 is a signaling molecule involved in photosynthesis modulation in rice (Bonifacio et al. 2011; Carvalho et al. 2014; Sousa et al. 2015) and this process is one of the most important associated with plant growth. Thus, changes in the mitochondrial H_2O_2 homeostasis associated with GPX abundance could trigger changes in chloroplasts involving a cross talk between these two organelles, with consequences on photosynthesis. As the major photosynthetic restriction in GPX1s plants was related to Calvin reactions and not to PSII activity, it is plausible that impairment in CO_2 assimilation could have occurred by enzymatic inhibition and/or downregulation in gene expression related to Calvin cycle and not related to photosynthetic electron transport chain. Photosynthetic regulation by change in a single gene expression addressed to mitochondria has been reported (Dutilleul et al. 2003; Sweetlove et al. 2006; Nunes-Nesi et al. 2007; Fuentes et al. 2011; Rzigui et al. 2013). These results are intriguing since the most of encoded proteins are not directly related to photosynthesis, as it is the case of mitochondrial isoform GPX1.

Recent works have shown crosstalk between respiration and photosynthesis (for a review, see Araújo et al. 2014). Tobacco mutants deficient in mitochondrial respiratory complex I activity exhibited impaired CO_2 assimilation (Rzigui et al. 2013). Additionally, it has been shown that the mitochondrial alternative oxidase (AOX) pathway is associated with the protection of the photosynthesis apparatus against photodamage (Dinakar et al. 2010; Zhang et al. 2012; Vishwakarma et al. 2014). Mitochondrial cytochrome oxidase (COX) activity was also associated with sustaining photosynthesis by ROS modulation (Dinakar et al. 2010; Blanco et al. 2014). Moreover, the lack of uncoupling proteins in knockout *Arabidopsis* is associated with lower CO_2 assimilation, higher non-photochemical quenching and reduced growth, suggesting that the control of electron flux in the mitochondrial electron transport chain is associated with photosynthesis performance (Sweetlove et al. 2006).

It has been also proposed in the last decades that excess reducing equivalents generated in chloroplasts can be dissipated by the export into mitochondria via NAD(P)H-dehydrogenases and malate-oxaloacetate shuttle (Scheibe et al. 2005; Araújo et al. 2014). In mitochondria, these reducing equivalents would be subsequently oxidized by the respiratory electron transport chains – ETC (Noctor et al. 2007), thus allowing the maintenance of high rates of photosynthesis by relieving electron pressure in chloroplasts. The AOX has been shown as an example of a sinker for these electrons transferred from the chloroplast to the mitochondria

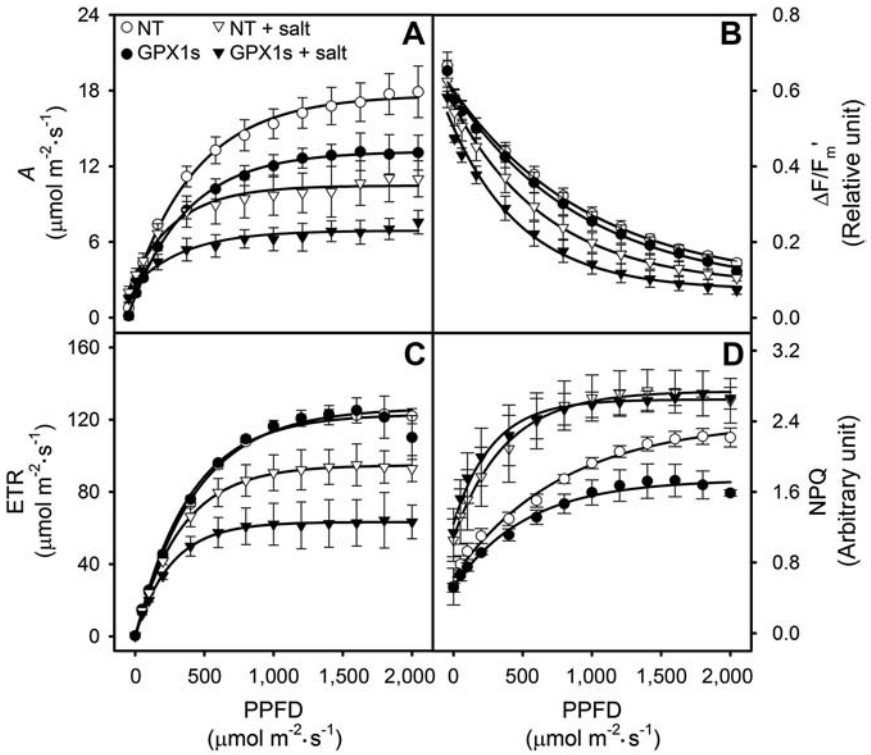

Figure 6. Photosynthetic efficiency parameters in leaves exposed to light
A-PPFD (**A**), $\Delta F/F_m'$-PPFD (**B**), ETR-PPFD (**C**) and NPQ-PPFD (**D**) curves in leaves of non-transformed (NT) and *GPX1* silenced rice exposed to control conditions and 100 mM NaCl for 6 d. The photosynthetic photon flux density (PPFD), VPD and temperature used in each measurement are described in the Materials and Methods section. Each point represents the mean of four replicates ± *SD*.

(Zhang et al. 2012). Moreover, mitochondrial ETC are a known source of ROS such as superoxide ($O_2^{\cdot-}$) and H_2O_2 (Hebelstrup and Møller 2015). Based on this and on the GPX function as a H_2O_2 scavenger, one would hypothesize that mitochondrial GPXs could also contribute to improve the photosynthetic efficiency by mitigating the excess energy in the chloroplast and mitochondrial electron transport chains.

Despite several works using genetic approaches having clearly evidenced that downexpression of proteins encoded to cytosol and peroxisomes in rice are able to deeply alter photosynthesis, the metabolic and genetic mechanisms that control such changes are poorly known (Carvalho et al. 2014; Sousa et al. 2015). Particularly, the general effects caused by overexpression (or knockout) of some encoded mitochondrial proteins on photosynthesis are well characterized. However, in opposition, the underlying mechanisms that control the molecular and the biochemical processes are poorly evidenced (Araújo et al. 2014). These problems are particularly important when plants are exposed to biotic and abiotic stresses. In these physiological conditions, the homeostasis is frequently disrupted, changing deeply gene and metabolic networks (Munné-Bosch et al. 2013). In the case of GPX1s plants exposed to salt stress, the transgenic line displayed a different response compared with salt-treated NT plants and with GPX1s cultivated under normal conditions.

Salinity causes several physiological disturbances in rice plants, especially a rapid decrease in the growth and impairment in photosynthesis by metabolic and stomatal limitations (Bonifacio et al. 2011). Under saline conditions, GPX1 is crucial for photosynthesis and root growth, perhaps because this mitochondrial isoform should trigger a different organ-dependent redox modulation. Recently, Passaia et al. (2014b), demonstrated that the *Arabidopsis AtGPX* gene family are involved with root growth and architecture but not in shoot development and that processes in roots are under hormone control. Thus, apparently GPX1 might display multiple physiological roles such as positive modulation on the whole plant growth under normal conditions and for root development under salinity. GPX1 is also important to improve water use efficiency in rice plants under salt stress, but in spite of this favorable characteristic, the expression of *GPX1* in NT plants was not able to improve its biomass production.

Under salt stress, the photorespiration is strongly decreased in silenced plants, clearly indicating that GPX1 would be important for this process. Photorespiration is very important in C3 plants under salinity and drought stress to dissipation of excess energy, avoiding photo-inhibition and minimizing oxidative stress (Lima Neto et al. 2014; Silva et al. 2015). Another important result of this study was the higher stomatal conductance displayed by transgenic GPX1s under normal conditions. The effects of *GPX1* silencing on rice stomata control was in agreement with the roles reported for some *Arabidopsis* chloroplastic GPX (Miao et al. 2006). Further studies are needed to elucidate the cross talk

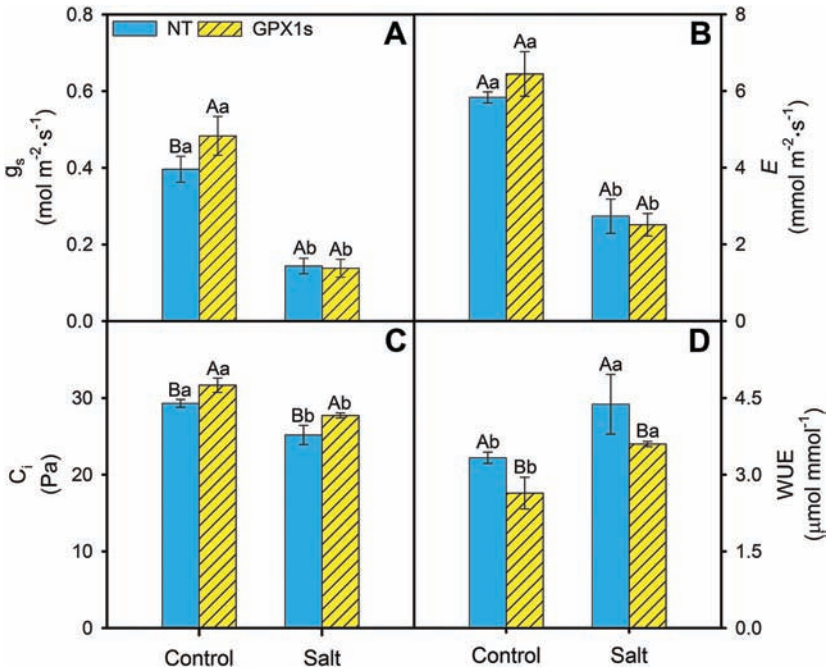

Figure 7. Gas exchange parameters in leaves exposed to salinity and control
Stomatal conductance (**A**), transpiration (**B**), internal CO_2 concentration (**C**) and water use efficiency (**D**) in leaves of non-transformed (NT) and *GPX1* silenced rice exposed to control conditions and 100 mM NaCl for 6 d. The means are representative of four replicates ± *SD*. Capital letters represent significant differences between genotypes within treatments, and lower case letters represent significant differences between treatments within genotypes, calculated by Tukey's test ($P < 0.05$).

mechanisms involved with the mitochondrial isoform GPX1 linking mitochondria and photosynthesis in order to reveal the metabolic role of this protein in photosynthetic efficiency. Moreover, new approaches are required to elucidate the main mechanisms involved in plant cell cross talk, especially between respiration and photosynthesis. Additionally, it is important to clarify the contribution of GPX1 in root growth under salinity.

In conclusion, the *OsGPX1* gene expression and its downstream metabolic and molecular changes are essential for rice growth and photosynthesis improvement in both non-stressful and salinity conditions. In the first condition, the photosynthesis impairment in GPX1 deficient plants occurs especially at level of Calvin cycle reactions and, in a minor extension, in photochemical phase. In opposition, under salinity, this mitochondrial isoform is important for both phases of photosynthesis, root growth, water use efficiency and photorespiration. As the positive role of GPX1 in the enhancement of growth and photosynthesis is related to increase in H_2O_2 and decrease in GSH, we have suggested that such physiological changes could be originated from redox alterations in mitochondria, which could alter chloroplast metabolism probably by a cross talk mechanism.

MATERIALS AND METHODS

Construction of plant vectors, plant transformation and plant growth conditions

The *GPX1* silenced rice plants were generated according previously described in Passaia et al. (2014a). A 115 bp sequence was amplified by PCR based on the sequence of the *OsGPX1* gene (LOC_Os04g46960). The primers used were 5′-CACCGTCCTCGTCTCCACGCTACC-3′ and 5′-AAGTCGTGGACG-GAGGTG-3′. The PCR product was cloned into the pANDA vector (Miki and Shimamoto 2004), which enables hairpin structure formation and post-transcriptional silencing of the *OsGPX1* gene. The pANDA vector contains the maize ubiquitin promoter and the *Hpt* gene for selection by hygromycin. The transformation of rice calli was achieved via *Agrobacterium tumefaciens*, as described previously (Upadhyaya et al. 2000). Regenerated seedlings were grown at 28 °C in MS medium with a photoperiod of 12 h and 150 μmol m^{-2} · s^{-1} of photosynthetic photon flux density (PPFD) in a growth chamber for 7 d. Three lines of mGPX1 transgenic plants were selected based on the transcript levels: L29, L36, and L40, which expressed 29%, 27%, and 39%, respectively, of the transcript amount of NT plants.

Plant growth

The L29, L36, and L40 lines and the NT plants (T1 generation) were transferred to 2 L plastic pots filled with half-strength Hoagland's solution. The pH was adjusted to 6.0 ± 0.5 every 2 d, and the nutrient solution was changed weekly. The seedlings were previously grown for 48 d in a greenhouse with the following conditions: day/night mean temperature of 29/24 °C, mean relative humidity of 68%, and a photoperiod of 12 h. The light intensity inside the greenhouse varied as for a typical day from 6:00 a.m. to 6:00 p.m., reaching an average maximum PPFD of 820 μmol m^{-2} · s^{-1} at noon.

Salt treatment and plant harvesting

48 d-old transformed and NT plants were grown as described previously. For the salt treatment, a group of plants was transferred to a nutrient solution supplemented with 100 mM NaCl, whereas the control was kept in the normal nutrient solution. The plants were grown for 6 d in the greenhouse as described previously. For photosynthesis measurements and harvesting, the plants were transferred to a growth chamber at 27 ± 2 °C/24 ± 2 °C (day/night) and 70 ± 10% relative humidity with a PPFD of 400 μmol $m^{-2} \cdot s^{-1}$ and a 12 h photoperiod and acclimated for 24 h. After photosynthetic measurements and electrolyte leakage analysis, the roots and leaves were harvested, frozen in liquid N_2, and stored at −85 °C for further biochemical and molecular characterization.

Quantitative real-time PCR determination

Quantitative real-time PCR assays were performed as described previously (Passaia et al. 2014a). The qRT-PCR experiments were carried out using cDNA synthesized from total RNA purified with TRIzol (Invitrogen, Carlsbad, CA, USA) as previously described (Passaia et al. 2014a). Primer-pairs to amplify *OsFDH3* gene (LOC_Os02g57040), rice 40S ribosomal protein S27a (LOC_Os01g22490) and *OseFa1* gene (LOC_Os03g08020) were used as internal controls to normalize the amount of mRNA present in each sample. All qRT-PCR reactions were performed with an Applied Biosystems StepOne plus Real-Time PCR system (Foster City, CA, USA) using SYBR-green intercalating dye fluorescence detection.

Protein extraction and glutathione peroxidase activity assay

Total soluble protein extracts were obtained from fresh leaves (150 mg) or roots (300 mg) that were ground to powder in a mortar in liquid N_2. Then, 1 mL of 100 mM potassium phosphate buffer (pH 7.5) was added. The extracts were centrifuged at 14,000 *g* for 15 min at 4 °C and the supernatants were collected. GPX activity was measured spectrophotometrically according to Liu et al. (2010), with modifications. Aliquots of 100 μL of total soluble protein extract were incubated with 4 mM GSH for 5 min and the reaction was started with the addition of 0.5 mM cumene hydroperoxide. The reaction was stopped with the addition of 100 mM TCA and incubation for 1 min. The remaining GSH was quantified according to Griffith (1980) and the activity was expressed in μmol GSH · g^{-1} FW · min^{-1}. The total soluble protein content was measured in the same enzymatic extract by the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as the standard.

Reduced glutathione, lipid peroxidation and hydrogen peroxide determination

Fresh leaf samples were macerated in liquid N_2 in the presence of 5% (w/v) TCA and centrifuged at 12,000 *g* (4 °C). The supernatant was immediately used for GSH and TBARS determination. The reduced glutathione content (GSH) was measured as described by Griffith (1980). The reduced glutathione content was measured by carrying out a reaction in the presence of 100 mM sodium phosphate buffer (pH 7.0) and 6 mM 5,5′-dithiobis-(2-nitrobenzoic acid). The absorbance was read at 412 ηm and the GSH content was expressed as μmol g^{-1} FW. The lipid peroxidation was measured based on the formation of thiobarbituric acid-reactive substances (TBARS) in accordance with Cakmak and Horst (1991). The concentration of TBARS was calculated using its absorption coefficient (155 mM^{-1} cm^{-1}), and the results were expressed as ηmol MDA-TBA g^{-1} FW. The H_2O_2 content was quantified in an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Life Technologies, Carlsbad, CA, USA). The absorbance was quantified spectrophotometrically using a wavelength of 560 ηm, and the H_2O_2 content was expressed as μmol H_2O_2 g^{-1} FW (Zhou et al. 1997).

Electrolyte leakage and Na^+ content

Membrane damage (electrolyte leakage) was measured as described previously by Blum and Ebercon (1981). Five leaf segments (5 cm length) were placed in test tubes containing 9 mL of deionized water. The flasks were incubated in a shaking water bath (25 °C) for 24 h and the electrical conductivity in the medium (M1) was measured. The segments were then boiled (95 °C) for 60 min and cooled to 25 °C and the electrical conductivity (M2) measured again. The electrolyte leakage (EL) was estimated using the relation: EL (%) = M1/M2 × 100. The Na^+ concentration was determined by flame photometry (Micronal, São Paulo, Brazil) from leaf powdered-lyophilized extract obtained after boiling at 100 °C in deionized water for 60 min as described previously (Cavalcanti et al. 2004). The Na^+ content was expressed as μmol Na^+ g^{-1} DW.

Determination of gas exchange and chlorophyll *a* fluorescence

The CO_2 assimilation rate (*A*), stomatal conductance (g_s), intercellular CO_2 partial pressure (C_i) and transpiration (*E*) were measured in fully expanded leaves from both non-transformed and *GPX1* knockdown rice plants with a portable infrared gas analyzer system equipped with an LED source and a leaf chamber (IRGA LI-6400XT, LI-COR, Lincoln, NE, USA). The internal parameters in the IRGA chamber during gas exchange measurements were 1,000 μmol $m^{-2} \cdot s^{-1}$ PPFD, 1.0 ± 0.2 kPa VPD and 38 Pa CO_2, at 28 °C. The *A* was measured in response to changes in PPFD and C_i. Each of these conditions was separately controlled inside the IRGA leaf chamber. The water use efficiency (WUE) was calculated as *A*/*E*. The *A*-PPFD and *A*-C_i fitting curves were determined according to models proposed by Lieth and Reynolds (1987) and Sharkey et al. (2007), respectively. The maximum carboxylation rate of Rubisco (Vc_{max}), the maximum electron transport rate (J_{max}), the dark respiration (R_d) and the photorespiration (P_r) were derived from the *A*-C_i curve, and the maximum photosynthetic rate (A_{max}) and the quantum efficiency (α) were calculated from the *A*-PPFD curve.

In vivo chlorophyll *a* fluorescence was measured using a LI-6400-40 Fluorometer (LI-COR, Lincoln, NE, USA) coupled with an IRGA equipment. The actinic light used to measure both gas exchange and chlorophyll *a* fluorescence was 1,000 PPFD. The fluorescence parameters were measured by the saturation pulse method (Schreiber et al. 1995) in leaves exposed to light and dark-adapted conditions. The intensity and duration of the saturation light pulse were 8,000 μmol m^{-2} s^{-1} and 0.7 s, respectively. The amount of blue light was set to 10% of the PPFD to maximize the stomatal aperture (Flexas et al. 2007). The maximum quantum yield of photosystem II (PSII) ($F_v/F_m = (F_m - F_o)/F_m$) was measured in dark-adapted conditions and the effective quantum yield of PSII (ΔF/

$F_m' = (F_m' - F_s)/F_m'$) was measured in leaves exposed to actinic light at 1,000 $\mu mol\ m^{-2} \cdot s^{-1}$ PPFD. The photochemical quenching coefficient was calculated by ($qP = (F_m' - F_s)/(F_m' - F_o')$), the non-photochemical quenching coefficient was calculated by ($NPQ = (F_m - F_m')/F_m'$), and the actual flux of electrons from PSII was calculated by ($ETR = (\Delta F/Fm' \times PPFD \times 0.5 \times 0.84)$). To evaluate the ETR, 0.5 was used as the fraction of excitation energy distributed to PSII and 0.84 was used as the fraction of incoming light absorbed by the leaves. The F_m and F_o parameters correspond to the maximum and minimum fluorescence of dark-adapted leaves, respectively; F_m' and F_s are the maximum and steady state fluorescence in the light-adapted leaves, respectively, and F_o' is the minimum fluorescence after far-red illumination of the previously light-exposed leaves (Genty et al. 1989; Schreiber et al. 1995; Flexas et al. 2007). The same fully expanded leaves were used for both photochemistry and gas exchange measurements.

Statistical analysis and experimental design

The experiments were conducted in two different experimental designs. The physiological and biochemical characterizations were carried out in completely randomized blocks with four genotypes (L29, L36 and L40 transgenic lines and NT plants) and four replicates. The salt stress experiment was performed in a 2×2 factorial design with two genotypes (NT and GPX1s) $\times$ 2 NaCl concentrations (0 and 100 mM) and four replicates. An individual pot containing two plants represented one replicate. The experiments were repeated three times and the presented results are representative of them all. The data were analyzed by ANOVA and the means were compared by Tukey's test at a confidence of 0.05.

ACKNOWLEDGEMENTS

The authors are grateful to Coordination for the Improvement of Higher Education Personnel (*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* - CAPES) and National Council for Scientific and Technological Development (*Conselho Nacional de Desenvolvimento Científico e Tecnológico* - CNPq) – Proc. 486231-2012-7 for financial support.

AUTHOR CONTRIBUTIONS

Y.L-M. conducted the experiments, performed the biochemical determinations and data organization, and participated in manuscript writing. F.E.L.C. contributed to writing and data interpretation. M.O.M. performed gas exchange measurements. G.P. realized qRT-PCR and obtained transformed plants. R.H.V.S. collaborated in experimental conduction. M.C. L.N. performed photochemical analysis. M.M-P. designed GPX1-hairpin structure and collaborated in data discussion, and J.A.G.S. conceived part of the experiments and wrote the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Phenotypic and molecular characterization of NT and three GPX1 plant lines Habitus of representative pots and the transcript amounts of the mitochondrial *OsGPX1* gene in non-transformed and silenced lines L29, L36 and L40 of rice plants at vegetative V10-V13 stages.

Figure S2. Morphological aspects of NT and GPX1 plant lines after exposure to salt and control Habitus of 45-day-old non-transformed and *GPX1* silenced rice plants exposed to control conditions and 100 mM NaCl for six days.

Figure S3. Relationship between CO_2 assimilation and intercellular CO_2 concentrations *A*-C_i curves in leaves of non-transformed (NT) and *GPX1* silenced rice exposed to control and 100 mM NaCl for six days.

Table S1. Phenotypic characterization of NT and three GPX1 plant lines

Parameters associated with the physiological characterization of *GPX1* silenced lines and non-transformed (NT) plants.